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Analysis of 3-*O*-sulfo group containing heparin tetrasaccharides in heparin by liquid chromatography-mass spectrometry

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Abstract

Complete heparin digestion with heparin lyase 2 affords a mixture of disaccharides and resistant tetra-saccharides with 3-*O*-sulfo group containing glucosamine residues at their reducing ends. Quantitative on-line liquid chromatography-mass spectrometric analysis of these resistant tetrasaccharides is described. The disaccharide and tetrasaccharide composition of seven porcine intestinal heparins and five low molecular weight heparins were analyzed by this method. These resistant tetrasaccharides account for from 5.3 to 7.3 wt% of heparin and from 6.2 to 8.3 wt% of low molecular weight heparin. Since these tetrasaccharides are derived from heparin's antithrombin III-binding sites, we examined whether this method could be applied to estimate the anticoagulant activity of heparin. The content of 3-*O*-sulfo group containing tetrasaccharides in a heparin positively correlated ($r = 0.8294$) to heparin's anticoagulant activity.

Keywords

3-*O*-sulfo group; tetrasaccharides; porcine intestinal heparin; anticoagulant activity; RPIP-LC-MS; heparin lyase 2

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Introduction

Heparin, a sulfated, linear polysaccharide, together with its low molecular weight derivatives, are widely used as clinical anticoagulants [1]. The heparin polysaccharide is comprised of a repeating, 1,4-linked disaccharide unit comprised of uronic acid (α -L-iduronic (IdoA) or β -D-glucuronic (GlcA) or and α -D-glucosamine (GlcN) [1,2]. Heparin, on average is comprised of 20 disaccharide repeating units, has an average molecular weight of ~12,000 and is polydisperse ($M_W/M_N > 1$) [3]. Heparin is also microheterogeneous with some of the uronic acid residues substituted with 2-O-sulfo groups and some of the GlcN residues substituted with 6-O-sulfo and or 3-O-sulfo groups and with N-sulfo groups, N-acetyl groups or N-unsubstituted [3,4].

The anticoagulant activity of heparin is primarily ascribed to its inhibition of the blood coagulation cascade [1]. It does so by binding through a pentasaccharide sequence to antithrombin III (AT) [5]. The bound AT undergoes a conformational change, which enhances its ability to inactivate thrombin and other serine protease inhibitors in the coagulation cascade. Since thrombin is ultimately responsible for the conversion of soluble fibrinogen into an insoluble fibrin clot, its inactivation by heparin-AT complex results in anticoagulation [6]. While the pentasaccharide AT-binding site is often described as a single sequence, it is actually a collection of sequences that fit a general motif consistent with the structure-activity relationship (SAR) first reported by Rosenberg and co-workers for the binding and activation of AT [7]. The sequence of this AT-binding site can vary based on the species/tissue source of a heparin [8,9]. Moreover, the residues surrounding this pentasaccharide sequence may also contain preferred motifs [8].

The biosynthesis of heparin and, thus, the construction of the AT-binding site takes place in the Golgi through the concerted action of glycosyltransferases, de-N-acetylase-N-sulfotransferases (NDSTs), various O-sulfotransferases (OSTs) and C5 epimerase (C5epi) [10–12]. While the control of these biosynthetic enzymes is not completely understood, much is known about the specific isoforms of the enzymes expressed in mast cells responsible for heparin biosynthesis and the required order of action of these enzymes [10–13]. The 3-O-sulfotransferase isoform-1 (3OST-1) is believed to act in the final step of heparin biosynthesis on precursor site(s) to form the AT-binding site(s) [14].

Pharmaceutical heparin is primarily derived from porcine intestinal tissues although some bovine lung heparin is still used [9]. Moreover, low molecular weight heparins (LMWHs), having average molecular weights of 6,000 to 8,000, which show improved bioavailability and pharmacodynamics are primarily derived from the controlled chemical or enzymatic depolymerization of porcine intestinal heparin [15]. Because of heparins high molecular weight and complex structure, many laboratories rely on the use of heparin lyase enzymes for the controlled depolymerization of heparin into oligosaccharides for detailed structural characterization [16,17]. Heparin lyases act as eliminases, cleaving the glycosidic linkage between GlcN and uronic acid, affording oligosaccharide products having an unsaturated uronic acid residue (UA) at their non-reducing ends [16,17]. Heparin lyase 1 cuts heparin through its highly sulfated domains, heparin lyase 3 cuts heparin through its domains having low sulfation and heparin lyase 2 can cut heparin at most sites within its structure [18–20].

None of these enzymes, however, can cut the glycosidic linkage between GlcN and uronic acid on the non-reducing side of a 3-*O*-sulfoglucosamine residue (Figure 1) [21–24]. Thus, even complete digestion of heparin with one or more heparin lyase results in some small amount of resistant 3-*O*-sulfo oligosaccharides in addition to the major disaccharide products [22, 23, 26]. In an early study [26] the content of a 3-*O*-sulfo group-containing hexasaccharide (UA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S), corresponding to one AT-binding site variant afforded on treatment with heparin lyase 1, was correlated to the anticoagulant activity of porcine intestinal heparins. Subsequently, a second AT-binding site variant in the form of a tetrasaccharide (UA2S-GlcNS6S-GlcA-GlcNS3S6S) was prepared using heparin lyase 1 but this time from bovine lung heparin [8]. More recently, three additional 3-*O*-sulfo group-containing tetrasaccharides were prepared from porcine intestinal using heparin lyase 2 and characterized [23]. Thus, it became clear that a new approach was required using heparin lyase 2, the least selective of the three heparin lyases [18,19], to prepare, separate, identify and quantify all of the AT-binding site variants from porcine intestinal heparin. Such a method might also allow an improved correlation of the content of 3-*O*-sulfo group-containing oligosaccharides to the anticoagulant activity of porcine intestinal heparins.

Materials and methods

Materials

Unsaturated heparin/HS disaccharide standards (0S: UA-GlcNAc, NS: UA-GlcNS, 6S: UA-GlcNAc6S, 2S: UA2S-GlcNAc, NS2S: UA2S-GlcNS, NS6S: UA-GlcNS6S, 2S6S: UA2S-GlcNAc6S, triS: UA2S-GlcNS6S) were obtained from Iduron Co (Manchester, UK). *E. coli* expression and purification of the recombinant *F. heparinum* heparin lyase 2 (no EC assigned) was performed in our laboratory as described [25]. Pharmaceutical heparin samples were from a variety of commercial suppliers and low molecular weight heparin (LWMH) samples, made through chemical β -elimination were obtained from Chinese generic manufacturer. Ion pairing reagent tributylamine was purchased from Sigma Aldrich (Saint Louis, Missouri, USA). All other chemicals were of HPLC grade.

Preparation of 3-*O*-sulfation tetrasaccharides standards

Preparation of 3-*O*-sulfated tetrasaccharide standards was described previously [23]. Briefly, 400 mg heparin sample was dissolved in 50 mM sodium phosphate buffer (pH 7.4) and was exhaustively digested by heparin lyase 2 (35 IU, activity against heparin). Buffer salts and disaccharides within the product mixture were removed by Bio-Rad P10 column (100 $\times$ 50 cm) eluted at 1.2 mL/min with 0.2 M NaCl. The remaining tetrasaccharides mixture was desalted on a Bio-Rad P2 column (100 $\times$ 20 cm) and lyophilized. The resulting mixture was fractionated on a semi-preparative strong anion exchange (SAX)-high performance liquid chromatography (HPLC) column Spherisorb S5 (20 $\times$ 250 mm Waters, Milford, MA, USA). A gradient of solution A (water, pH 3.5, adjusted with HCl) for 2 min and followed by a linear gradient 0–60% solution B (2.0 M NaCl, pH 3.5, adjusted with HCl) from 2 to 60 min was used at flow rate of 4.0 mL/min with absorbance detected at 232 nm. Individual peaks were desalted on a Bio- Rad P2 column and were further purified by repeated separation on

the same SAX-HPLC column. The purified tetrasaccharide standards were quantified by carbazole analysis described previously [31].

Enzymatic digestion of heparin and LMWH

The heparin and LMWH samples (100 µg) were dissolved in 100 µL of distilled water and digested by heparin lyase 2 (20 mU) at 35°C for 10 h. Aliquots were immediately heated in a 100 °C water bath to stop the reaction, and were then dried by lyophilization.

RPIP-HPLC-MS

LC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with 6300 ion trap. The column used was a Poroshell 120 C18 column (2.1 × 150 mm, 2.1 µm, Agilent Technologies, Inc. Wilmington, DE). Eluent A was water/acetonitrile (85:15) v/v, and eluent B was water/acetonitrile (35:65) v/v. Both eluents contained 12 mM TrBA and 38 mM NH₄OAc with pH adjusted to 6.5 with HOAc. A gradient of solution A for 2 min and followed by a linear gradient 0–40% solution B from 2 to 30 min, 40–50% solution B from 30 to 60 min was used at flow rate of 100 µL/min. The column effluent entered the source of the ESI-MS for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of –40.0 V, a capillary exit of –40.0 V, and a source of temperature of 350 °C, to obtain the maximum abundance of the ions in a full scan spectrum (200–1500 Da). Nitrogen was used as a drying (8 L/min) and nebulizing gas (40 psi).

Calibration

Quantification analysis of disaccharides and tetrasaccharides was performed by using calibration curves constructed by separation of increasing amounts of disaccharide and tetrasaccharide standards (10, 25, 50, 100, 150 and 200 ng per each disaccharide/tetrasaccharide). The linearity based on amount of disaccharide and peak intensity in mass spectrometry. All analyses were performed in triplicate.

Results and discussion

Depolymerization of heparin and LMWH using heparin lyase 2

Previous studies demonstrated that heparin lyase 2 exhibits a wide range of specificities acting on both heparin and heparan sulfate [18, 19]. It acts at linkages containing either (1→4)-α-L-iduronic acid or (1→4)-β-D-glucuronic acid residues and accommodates many sulfation modifications in these polysaccharides. When porcine heparin is exhaustively treated with heparin lyase 2 in addition to the disaccharides formed, some lyase resistant tetrasaccharides are observed [23] and mass spectral properties, the structures of these lyases resistant tetrasaccharides are shown in Table 1 and Figure 1. Each tetrasaccharide T1 (UA-GlcNAc6S-GlcA-GlcNS3S), T2 (UA-GlcNAc6S-GlcA-GlcNS3S6S) and T3 (UA-GlcNS6S-GlcA-GlcNS3S6S), has a 3-O-sulfo group containing glucosamine residue at its reducing end.

Method improvement for LC-MS analysis of porcine intestinal heparin 3-O-sulfo tetrasaccharides

SAX-HPLC with UV detection, using heparin lyases for mapping of heparin disaccharides and oligosaccharides [23, 26], failed to provide information on the structural diversity of 3-*O*-sulfo oligosaccharides, was relatively insensitive, and required high salt concentrations precluding its use with MS detection. Reversed-phase ion-pair (RPIP)-HPLC, an increasingly popular method for the separation of glycosaminoglycan oligosaccharides, relies on volatile lipophilic ion-pairing reagents in the mobile phase that play a critical role as mobile-phase modifiers, aiding in the retention and resolution of charged species on hydrophobic stationary phases [27,28]. RPIP-HPLC with UV detection at 232 nm has been well established for the profiling of disaccharides and oligosaccharides of porcine intestinal heparin and LMWHs digested by heparin lyases [29]. Due to the sensitivity of detection, only the main peak (UA-GlcNAc6S-GlcA-GlcNS3S6S) was detected and the other two compounds were out of the limit of detection [29]. RPIP-HPLC separation coupled to MS detection enhanced the detection sensitivity by an order of magnitude and offered a second method of analyte identification [30]. In our laboratory, RPIP-UPLC-MS has been applied successfully for analysis of chondroitin sulfate (CS)/dermatan sulfate (DS) disaccharides [31] and heparin/heparan sulfate (HS) disaccharides [32]. The quantification method of heparin/HS disaccharide analysis was established by RPIP-UPLC-MS and 12 mM TrBA/ 50mM ammonium acetate ion-pairing agent with a 1.7- μ m particle size Acquity UPLC BRH C18 column [32]. The same ion-pairing agent was applied for the analysis of porcine heparin 3-*O*-sulfo tetrasaccharides, except a Poroshell 120 C18 column (2.1 $\times$ 150 mm, 2.1 μ m, Agilent Technologies, Inc. Wilmington, DE) was used instead of the UPLC BRH C18 column, as this column provides high efficiency at lower pressures and is ideal for fast or high resolution separations of many types of analytes. Eight heparin/HS disaccharides and three tetrasaccharides were separated and detected by the extracted chromatography (EIC) (Figure 2). ESI-MS detection affords the mass of each tetrasaccharide analyte (Figure 2, inset). Peaks were observed at m/z 477.1 (UA-GlcNAc6S-GlcA-GlcNS3S), m/z 517.1 (UA-GlcNAc6S-GlcA-GlcNS3S6S), and m/z 537.1 (UA-GlcNS6S-GlcA-GlcNS3S6S), and all of these are double charged ions.

Standard curve for tetrasaccharide analysis

The three 3-*O*-sulfated tetrasaccharide standards were prepared in our laboratory according to the method described previously [23]. The accurate amount of each tetrasaccharide was determined by carbazole analysis [33]. An equal-mass mixture of the three tetrasaccharide standards (10, 25, 50, 100, 150 and 200 ng per each tetrasaccharide) were analyzed by RPIP-LC-MS to evaluate the sensitivity and linearity of tetrasaccharide amount and the peak intensity using EIC detection. The integrated tetrasaccharide peak areas show good linearity when plotted as a function of their amount (Figure 3). The different slopes of these curves reflect the different ionization efficiencies for the corresponding tetrasaccharides in an ESI source [32]. Notably, T3 with five sulfo groups showed a greater capacity to ionize, whereas T1 with three sulfo groups had the lowest ionization efficiency under these experimental conditions.

LC-MS analysis of porcine heparins and LMWHs

Seven commercial pharmaceutical heparin samples and five LMWH samples were completely digested using heparin lyase 2 in triplicate and then analyzed by RPIP-LC-MS. All the disaccharides and tetrasaccharides can be detected and quantified by this method. Their mass ratio provides a fingerprint of the heparin from which they are derived. The extracted ion chromatogram (EIC) showing their disaccharide and tetrasaccharide composition are presented in Figure 4 and Figure 5. All the eight disaccharides and three tetrasaccharides were detected in all samples; their quantity was calculated from a linear equation derived from a calibration curve of these eight disaccharide and three tetrasaccharide standards, and their compositions were given in Table 2. As expected, the major disaccharide in the heparin and LMWH is trisulfated disaccharide (UA2S-GlcNS6S, TriS). However, while the trisulfated disaccharide content in the heparin samples ranges from 74.5% to 81.6 %, it ranges from 80.9% to 84.5% in the LMWHs.

The 3 major 3-*O*-sulfo tetrasaccharides in the heparin and LMWH samples were quantified for the first time [30]. The tetrasaccharide composition of the heparin and LMWH samples are presented in Table 2. All the three tetrasaccharides were detected in the twelve samples analyzed. The major tetrasaccharide was T2 (UA-GlcNAc6S-GlcA-GlcNS3S6S) followed by T1 (UA-GlcNAc6S-GlcA-GlcNS3S) and T3 (UA-GlcNS6S-GlcA-GlcNS3S6S), which was present at less than 0.6 %. The total tetrasaccharide content in the heparin samples ranged from 5.3 to 7.3, whereas it ranged from 6.2 to 8.3 in the LMWHs.

Correlation between anticoagulant activity and 3-*O*-sulfo tetrasaccharide content of porcine heparins

The anticoagulant activity of heparin is primarily ascribed to the central 3-*O*-sulfo group containing glucosamine residue within the AT-binding site (Figure 1) pentasaccharide. In our previous study, the anticoagulant activity of AT-affinity-fractionated porcine intestinal heparin was found to correlate with the content of a 3-*O*-sulfo group-containing hexasaccharide sequence (UA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S) generated by heparin lyase 1 digestion [26]. A second 3-*O*-sulfo group-containing oligosaccharide was subsequently identified from heparin lyase 1 treated bovine lung heparin as UA2S-GlcNS6S-GlcA-GlcNS3S6S [8]. Moreover, three additional 3-*O*-sulfo group-containing tetrasaccharides were prepared and characterized from heparin lyase 2 treated porcine intestinal heparin [23]. The 3-*O*-sulfo group-containing hexasaccharide (UA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S), prepared from porcine intestinal heparin using heparin lyase 1, contains a tetrasaccharide substructure corresponding to T2 (UA-GlcNAc6S-GlcA-GlcNS3S6S), prepared from porcine intestinal heparin using heparin lyase 2. This suggests that porcine intestinal heparin might contain three to four AT-binding site structural variants. Since heparin lyase 2 has the lowest specificity of the three known *Flavobacterium* heparin lyases [18,19], it provides a relatively simple mixture of products [23], and is able to cleave heparin at both its highly sulfated and undersulfated domains, we selected this enzyme to digest porcine intestinal heparins. Interestingly, we observed only three of the four previously reported structural variants (T1, T2, and T3) and fail to observe the bovine lung heparin prepared variant (UA2S-GlcNS6S-GlcA-GlcNS3S6S). This may be due to its absence in some or in all porcine intestinal heparins.

We hypothesized that the 3-*O*-sulfated tetrasaccharide content obtained using heparin lyase 2 would reflect all the AT-binding site structural variants in porcine intestinal heparin and that these could be sensitively analyzed by RPIP-LC-MS and would correlate well to the anticoagulant activity of a porcine intestinal heparin sample. To test this hypothesis, the anticoagulant activity provided by the manufacturers was plotted as a function of 3-*O*-sulfo tetrasaccharide content of porcine heparins (Figure 6). There was a clear positive correlation ($r = 0.8294$) between anticoagulant activity and 3-*O*-sulfo tetrasaccharide content. These data indicate that 3-*O*-sulfo tetrasaccharide content of heparin can be considered as an index to evaluate the anticoagulant activity of heparin. The anti-IIa activity for LMW-heparins was not examined since these are principally anti-Xa agents and their reduced chain lengths (many chains are below the 16-saccharide units required for anti-IIa activity), complicates making such a correlation.

Conclusions

A quantitative on-line LC-MS analysis method has been developed that allows the analysis of 3-*O*-sulfo group containing tetrasaccharides in the heparin are afforded through digestion with heparin lyase 2, along with eight disaccharides. The 3-*O*-sulfo group containing tetrasaccharide standards were prepared and firstly applied to quantify their content in the porcine intestinal heparin samples. This method of analysis provides a profile of all the oligosaccharides present in heparin and LMWHs. The correlation between anticoagulant activity and 3-*O*-sulfo tetrasaccharide content of porcine heparins indicate that 3-*O*-sulfo tetrasaccharide content of heparin might be useful for evaluating anticoagulant activity.

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Abbreviations used

GlcA	β-D-glucuronic acid
IdoA	α-L-iduronic acid
GlcN	α-D-glucosamine
HS	heparan sulfate
CS	chondroitin sulfate
DS	dermatan sulfate
M_w	weight average molecular Weight
M_n	number average molecular weight
AT	antithrombin III
NDST	de- <i>N</i> -acetylase- <i>N</i> -sulfotransferase
LMWH	low molecular weight heparin

TrBA	tributylamine
LC-MS	liquid chromatography-mass spectrometry
SAX-HPLC	strong anion exchange-high performance liquid chromatography
RPIP	reversed-phase ion-pair
EIC	extracted ion chromatograph
ESI	electrospray ionization

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Fig. 1.

Structure of heparin, its AT-pentasaccharide binding site and its digestion by heparin lyase 2 to form disaccharides and three resistant tetrasaccharides (T1, T2 and T3) having 3-*O*-sulfo group containing glucosamine residues at their reducing ends.

Fig. 2.

The extracted ion chromatograms (EICs) and mass spectrogram of disaccharide and tetrasaccharide analysis of porcine heparin. (A) EICs of disaccharide and tetrasaccharide analysis of porcine heparin. Inserts show (B) EIC and mass spectrogram of Tetrasaccharide 1(T1, UA-GlcNAc6S-GlcA-GlcNS3S); (C) EIC and mass spectrogram of Tetrasaccharide 1(T2, UA-GlcNAc6S-GlcA-GlcNS3S6S); and (D) EIC and mass spectrogram of Tetrasaccharide 1(T3, UA-GlcNS6S-GlcA-GlcNS3S6S).

Fig. 3.
Standard curve prepared using three tetrasaccharide standards.

Fig. 4.
EICs of disaccharide and tetrasaccharide analysis of seven porcine heparins.

Fig. 5.
EICs of disaccharide and tetrasaccharide analysis of five LMWHs.

Fig. 6. Correlation between anticoagulant activity and 3-*O*-sulfo tetrasaccharide content of porcine heparins ($r = 0.8294$).

Table 1

Structure and mass of disaccharides and tetrasaccharides derived from porcine heparin by heparinase 2 digestion.

Peak Name	Found ions* (charge)	Calculated Mol Mass	Theoretical Mol Mass	Structure
Disaccharides				
0S	377.8 (-1)	378.8	379.1	UA-GlcNAc
NS	415.7 (-1)	416.7	417.1	UA-GlcNS
6S	457.8 (-1)	458.8	459.1	UA-GlcNAc6S
2S	457.8 (-1)	458.8	459.1	UA2S-GlcNAc
NS6S	496.1(-1)	497.1	497.0	UA-GlcNS6S
NS2S	496.1(-1)	497.1	497.0	UA2S-GlcNS
2S6S	537.9(-1)	538.9	539.0	UA2S-GlcNAc6S
Tri S	575.7(-1)	576.7	576.9	UA2S-GlcNS6S
Tetrasccharides				
T1	477.1(-2)	956.2	956.1	UA-GlcNAc6S-GlcA-GlcNS3S
T2	517.1(-2)	1036.2	1036.0	UA-GlcNAc6S-GlcA-GlcNS3S6S
T3	536.1(-2)	1074.2	1074.0	UA-GlcNS6S-GlcA-GlcNS3S6S

Table 2

Compositional analysis of commercial porcine heparins and LMWHs

Sample	Composition (%)										
	OS	NS	6S	2S	NS6S	NS2S	6S2S	TrIS	T1	T2	T3
Heparin 1	0.8±0.11	0.9±0.22	0.8±0.15	0.4±0.01	7.8±0.28	7.0±0.35	0.5±0.12	74.5±0.99	2.5±0.31	4.5±0.09	0.3±0.04
Heparin 2	1.1±0.23	1.3±0.18	0.8±0.08	0.5±0.06	5.4±0.67	5.5±0.23	0.5±0.07	78.0±0.98	2.5±0.54	4.0±0.25	0.4±0.01
Heparin 3	1.0±0.35	1.4±0.12	1.2±0.17	0.6±0.12	6.7±0.57	3.7±0.18	0.5±0.10	78.9±0.77	2.2±0.77	3.6±0.15	0.2±0.02
Heparin 4	1.0±0.32	1.2±0.05	1.3±0.08	0.9±0.07	5.3±0.32	4.2±0.33	0.5±0.09	79.5±0.56	2.1±0.56	3.8±0.07	0.2±0.01
Heparin 5	0.8±0.12	1.1±0.05	0.8±0.09	0.5±0.02	7.3±0.26	7.1±0.23	0.4±0.10	74.8±0.69	2.8±0.78	4.2±0.04	0.2±0.01
Heparin 6	0.8±0.29	2.1±0.15	2.2±0.05	1.3±0.06	3.8±0.44	1.8±0.24	1.1±0.14	81.6±1.21	2.0±0.34	3.0±0.12	0.3±0.01
Heparin 7	0.4±0.23	1.2±0.08	1.1±0.12	0.8±0.04	4.8±0.23	3.8±0.27	2.6±0.09	79.7±0.87	2.2±0.89	3.2±0.13	0.2±0.01
LMWH1	0.8±0.47	1.6±0.25	1.6±0.19	0.8±0.12	3.9±0.54	1.7±0.32	0.4±0.21	80.9±1.41	1.8±0.25	5.9±0.89	0.4±0.03
LMWH2	0.4±0.38	1.2±0.30	1.1±0.13	0.7±0.08	7.8±0.23	2.1±0.28	0.3±0.18	82.9±1.21	2.5±0.64	5.6±1.11	0.4±0.01
LMWH3	0.5±0.32	1.4±0.22	1.1±0.05	0.7±0.09	3.1±0.18	1.9±0.27	0.6±0.15	84.5±1.69	2.2±0.21	3.6±0.09	0.4±0.05
LMWH4	0.4±0.45	1.3±0.18	1.0±0.12	0.7±0.11	3.0±0.68	1.9±0.31	0.5±0.33	84.4±1.54	1.8±0.18	4.7±0.11	0.3±0.07
LMWH5	0.3±0.32	1.5±0.20	1.1±0.18	0.7±0.15	3.2±0.44	1.9±0.18	0.4±0.17	81.7±0.98	2.7±0.43	5.9±0.98	0.6±0.09